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Stephaochratidin A, a Rare Stephacidin-Asperochratide Hybrid with Ferroptosis Inhibitory Activity from the Deep-Sea-Derived *Aspergillus ochraceus*

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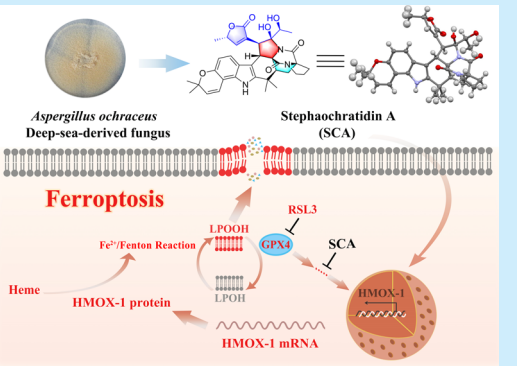
ABSTRACT: One rare stephacidin-asperochratide hybrid, stephaochratidin A (**1**), was isolated from the deep-sea-derived *Aspergillus ochraceus* MCCC 3A00521. The relative structure of **1** was determined by comprehensive analyses of its 1D and 2D NMR data as well as HRESIMS data. And the absolute configuration was unambiguously assigned by ECD calculations and the X-ray single-crystal diffraction analysis. Plausible biosynthetic pathway of **1** was proposed. Stephaochratidin A (**1**) exhibited significant ferroptosis inhibitory activity with the EC_{50} value of 15.4 μM by downregulating HMOX-1 expression and lipid peroxidation.



Prenylated indole alkaloids are characterized as diketopiperazine or bicyclo[2.2.2]diazaoctane rings, which are derived from tryptophan, proline, and isoprene units.[1] Prenylated indole alkaloids are characteristic metabolites produced by the fungi *Aspergillus* and *Penicillium*, and contain various skeletons such as stephacidin, brevianamide, paraherquamide, asperparaline, marcfortines, sclerotiamide, malbrancheamide, avrainvillamide, and notoamide.[2] Prenylated indole alkaloids are well-known for their remarkable cytotoxicity. For example, stephacidin B showed potent cytotoxicity against various human tumor cells with IC_{50} values ranged from 0.06 to 0.46 μM;[3] 6-*epi*-avrainvillamide exhibited strong cytotoxicity against HL-60 and A549 cells with IC_{50} values of 1.88 and 1.92 μM, respectively;[1b] sclerotiamide C possessed effective cytotoxicity against HeLa, A549, HepG2, and SMMC7721 cells with an IC_{50} value of approximately 1.6 μM.[1e] Due to their unique skeletons and remarkable cytotoxicity, prenylated indole alkaloids have become a fascinating target for total synthesis and biosynthesis.[4]

In the course of our ongoing research for structurally novel and biological significant secondary metabolites from deep-sea-derived fungi,[5] the strain *Aspergillus ochraceus* MCCC 3A00521 isolated from the Northeastern Pacific at a depth of −1334 m was chosen for systematic chemical investigation because of the potent ferroptosis inhibitory activity of its crude extract. As a result, one rare stephacidin, stephaochratidin A (**1**, Figure 1) was obtained, together with two known analogues, stephacidin A (**2**)[3] and notoamide E (**3**, Figure S1).[1a] Herein, we report the isolation, structure, and biological effects of these isolates.

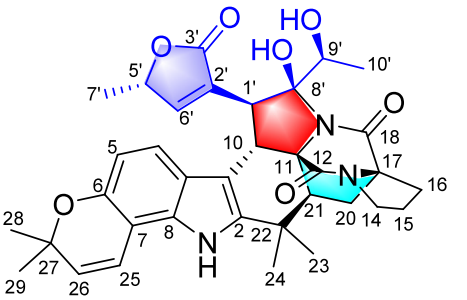


Figure 1. Chemical structure of stephaochratidin A (**1**).

Stephaochratidin A (**1**) was obtained as colorless crystals. Its molecular formula of $C_{35}H_{39}N_3O_7$ with 18 degrees of unsaturation was established by HRESIMS (*m*/*z*: $[M + Na]^+$ calcd for $C_{35}H_{39}N_3O_7Na$ 636.2686; found 636.2671). Detailed analyses of ^1H and ^{13}C NMR data implied that compound **1** (Table 1) possessed a stephacidin fragment,[3] except for the

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Stephaochratidin A (1) in DMSO-d_6 (J in Hz)

position	δ_C, type	δ_H, mult (J in Hz)
1		10.57 s
2	140.9, C	
3	106.2, C	
4	119.5, CH	6.80 d (8.5)
5	108.0, CH	6.32 d (8.5)
6	147.2, C	
7	104.8, C	
8	132.9, C	
9	121.4, C	
10[a]	39.1, CH	4.02 d (12.0)
11	67.5, C	
12	167.5, C	
14	43.5, CH_2	3.33 m
15	24.2, CH_2	2.03 m; 1.86 m
16	28.7, CH_2	2.59 m; 1.86 m
17	67.6, C	
18	168.6, C	
20	29.6, CH_2	2.13 dd (13.2, 10.2); 1.95 dd (13.2, 5.3)
21	44.8, CH	2.77 dd (10.2, 5.4)
22	34.2, C	
23	21.4, CH_3	0.98 s
24	27.6, CH_3	1.34 s
25	118.0, CH	6.95 d (9.8)
26	128.9, CH	5.72 d (9.8)
27	75.1, C	
28	27.3, CH_3	1.37 s
29	27.2, CH_3	1.36 s
1′	44.8, CH	3.27 d (11.9)
2′	130.8, C	
3′	173.4, C	
5′	78.0, CH	5.23 m
6′	155.5, CH	7.97 s
7′	17.4, CH_3	1.57 d (6.9)
8′	93.9, C	
9′	65.0, CH	4.35 m
10′	17.9, CH_3	0.81 d (6.5)
8′-OH		6.39 s
9′-OH		5.02 d (6.3)

[a]Observed in HMBC and HSQC spectra.

additional presence of one olefinic quaternary carbon at δ_C 130.8 (C-2′), one olefinic methine [δ_H 7.97 (s, 1H, H-6′), δ_C 155.5 (C-6′)], one extra carbonyl carbon at δ_C 173.4 (C-3′), two methyls [δ_H 1.57 (d, 3H, J = 6.9 Hz, 7′-Me), δ_H 0.81 (d, 3H, J = 6.5 Hz, 10′-Me); δ_C 17.4 (C-7′), δ_C 17.9 (C-10′)], three methines [δ_H 3.27 (d, 1H, J = 11.9 Hz, H-1′), δ_H 5.23 (m, 1H, H-5′), δ_H 4.35 (m, 1H, H-9′); δ_C 44.8 (C-1′), δ_C 78.0 (C-5′), δ_C 65.0 (C-9′)], and one nonprotonated carbon at δ_C 93.9 (C-8′). The HMBC correlations from H-7′ to C-5′ and C-6′; H-5′ to C-2′; H-6′ to C-3′; H-1′ to C-2′, C-3′, C-6′, C-8′, C-3, and C-10; H-10′ to C-8′ and C-9′ as well as the COSY cross-peaks of H-7′/H-5′/H-6′ and OH-9′/H-9′/H-10′ suggested a C_9 polyketide fragment containing an α,β-unsaturated γ-lactone ring. Thus, the planar structure of **1** was elucidated, which possessed a stephacidin-asperochratide hybrid scaffold (Figure 2).

In the NOESY spectrum, correlations were found of H-6′ to H-4/H-10/8′-OH, H-4 to H-10/8′-OH, 8′-OH to H-10, and H-1′ to H-21/10′-Me, indicating the relative configuration of

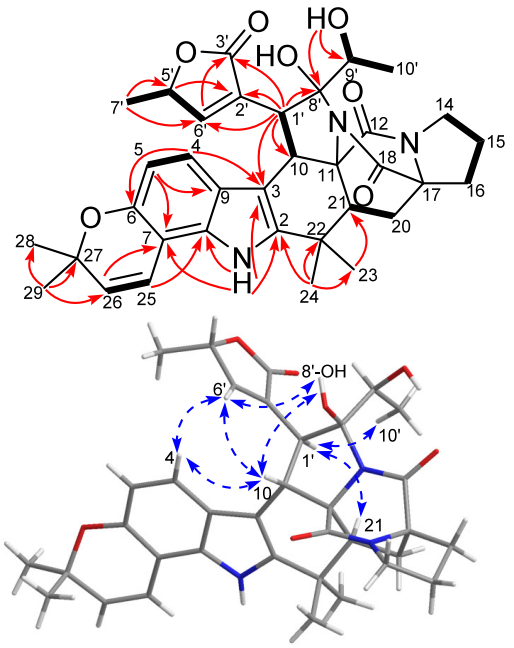

Figure 2. Key HMBC (red arrows) and NOESY (blue arrows) correlations of stephaochratidin A (**1**).

C-10, C-21, C-1′, C-8′, and C-9′ (Figure 2). However, the stereochemistry of C-11, C-17, and C-7′ could not be determined by NOE experiment. To further confirm the structural correctness of **1**, a suitable single crystal was successfully obtained from a mixed solvent system (MeOH/H_2O, 98:2) with a slow evaporation method and measured on an XtaLAB Pro: Kappa single diffractometer using Cu Kα radiation. The X-ray diffraction experiment (Figure 3) further

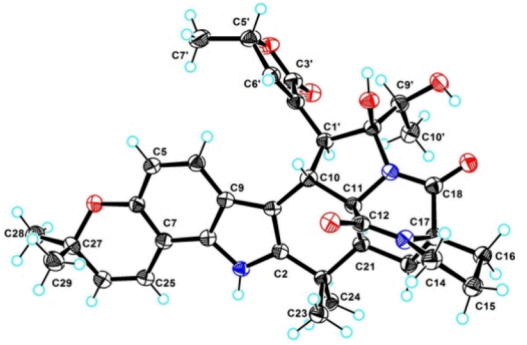

Figure 3. X-ray crystallographic structure of stephaochratidin A (**1**) with 30% ellipsoid contour probability.

certified the fantastic structure [Flack parameter 0.04(7)], and the absolute configuration of **1** was finally determined as 10*S*,11*R*,17*S*,21*S*,1′*S*,5′*S*,8′*S*,9′*S*, which was also supported by comparison of the experimental and calculated ECD spectra (Figure 4).

The biosynthetic pathway to stephaochratidin A (**1**) is proposed as illustrated in Scheme 1. A prior study has demonstrated the utilization of L-malic acid by a fungal PKS-NRPS synthetase TraA to construct the butyrolactone ring in the biosynthesis of terrestric acid.[6] In this study, we propose a similar PKS-NRPS pathway capable of utilizing L-malic acid, acetyl-CoA, and malonyl-CoA to generate the aldehyde intermediate (**i**). Subsequently, intermediate (**i**) can undergo

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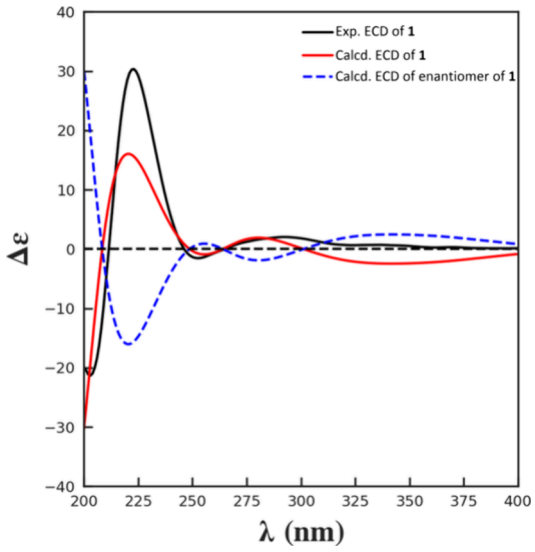


Figure 4. Experimental and calculated ECD spectra of **1**.

Knoevenagel condensation to yield butyrolactone intermediate (**ii**). The key block (**iii**) can be formed from the intermediate (**ii**) via a series of decarboxylation, reduction, and dehydration reactions. Meanwhile, deprotonation of a common fungal secondary metabolite stephacidin A (**2**) can afford the reactive intermediate **iv**, namely aspergamide B.[7] Ultimately, **1** could be formed through nucleophilic addition between intermediates (**iii**) and (**iv**) followed by a hydroxylation, which is similar to the cyclization mechanism in the biosynthesis of cyclopiazonic acid.[8]

Stephaochratidin A (**1**) was tested for inhibitory activity against glutathione peroxidase 4 inhibitor RSL3-induced ferroptosis. It showed potent effect on human melanoma cell A375, human renal cell carcinoma cell 786-O, and human nonsmall cell lung cancer cell H1299, with the EC_{50} value of 15.4 μM in A375 cells. Interestingly, **1** could also significantly inhibit ferroptosis triggered by system x_c-inhibitor erastin and iron oxidizer $FINO_2$, which induced ferroptosis through different mechanisms,[9] indicating **1** could be a common inhibitor of ferroptosis (Figure 5).

Ferroptosis is a type of regulated cell death in an iron and lipid peroxidation dependent manner.[10] Then, we tested the bioactivities of **1** as an iron chelator and antioxidant. As shown in Figure 6A–B, compound **1** did not exhibit activities as an iron chelator and antioxidant. In contrast, compound **1** effectively inhibited RSL3-induced lipid peroxidation (Figure 6C).

To further explore the mechanism of how stephaochratidin A (**1**) regulates ferroptosis, **1** was further examined on the expression of ferroptosis-related genes. As a result, **1**

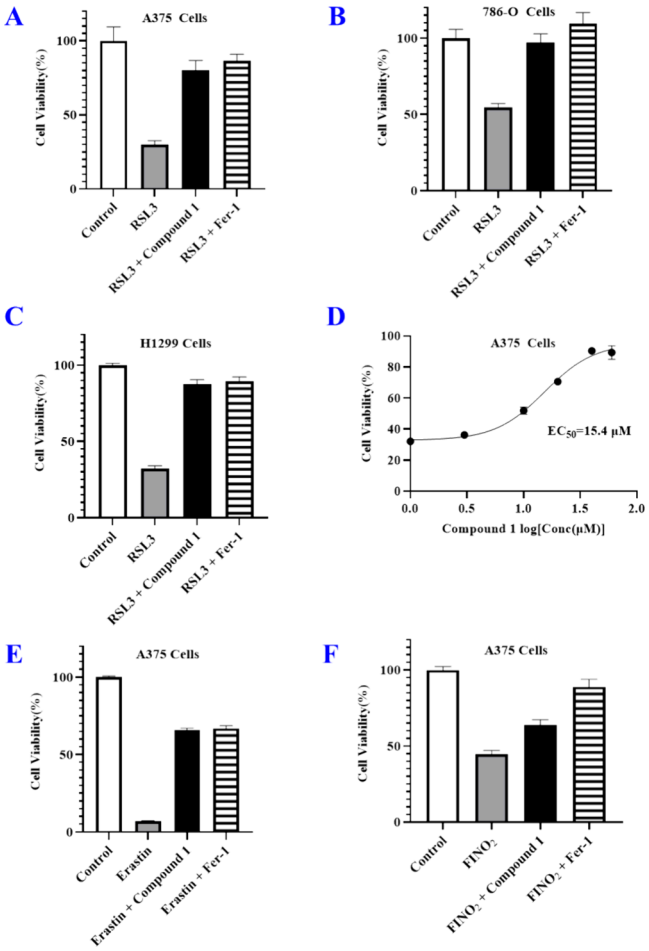
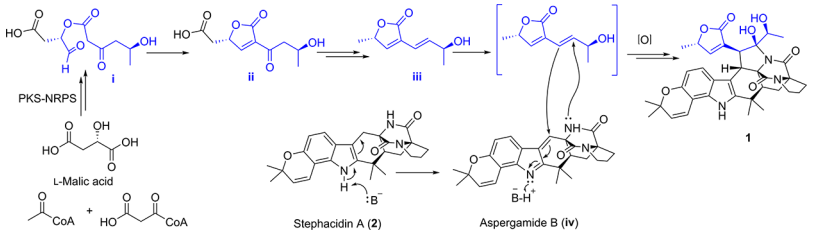


Figure 5. Stephaochratidin A (**1**) is a common ferroptosis inhibitor. (A–C) Compound **1** significantly inhibited RSL3-induced ferroptosis in A375, 786-O, and H1299 cells. (D) Compound **1** suppressed RSL3-triggered ferroptosis in a dose dependent manner. (E–F) Compound **1** remarkably prohibited erastin or $FINO_2$-stimulated ferroptosis in A375 cells.

attenuated both the protein and mRNA level of HMOX-1 induced by RLS3 (Figure 6D–E). HMOX-1 has been implicated in ferroptosis.[11] Consistent with previous studies,[11,12] inhibition of the enzymatic activity of HMOX-1 by small-compound ZnPP was able to inhibit ferroptosis (Figure 6F). Therefore, **1** could inhibit ferroptosis by suppressing HMOX-1 expression and lipid peroxidation induced by RSL3.

In summary, one novel stephacidin-asperochratide hybrid (stephaochratidin A, **1**) and two known analogues (stephacidin A, **2**; notoamide E, **3**) were isolated from the deep-sea-derived fungus *Aspergillus ochraceus* MCCC 3A00521. Stephaochratidin A (**1**) inhibited RSL3-induced ferroptosis with the EC_{50}

Scheme 1. Biosynthetic Proposal for Stephaochratidin A (1)



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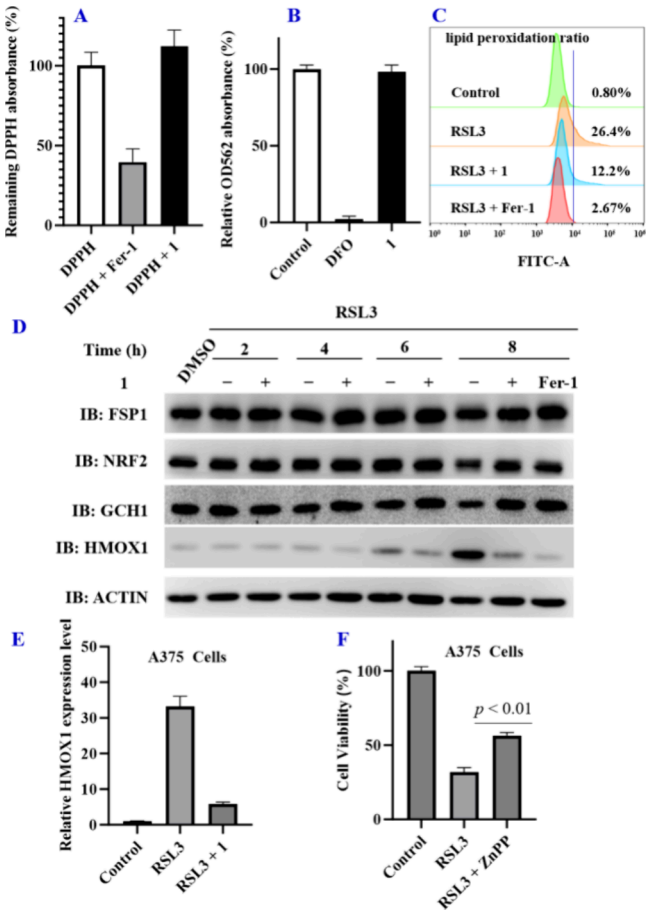

Figure 6. Stephaochratidin A (**1**) inhibited RSL-3-triggered ferroptosis by suppressing HMOX-1 expression and lipid peroxidation. (A) Compound **1** exhibited no radical scavenging activity on DPPH. (B) Compound **1** showed no iron chelating activity. (C) Compound **1** reduced lipid peroxidation. (D–E) Compound **1** inhibited HOMX-1 protein and mRNA level. (F) ZnPP significantly suppressed RSL3-induced ferroptosis.

value of 15.4 μM by suppressing HMOX-1 expression and lipid peroxidation.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.4c01745.

General experimental procedures, fungal material, fermentation, extraction and isolation, the quantum chemical calculations, bioassays, NMR spectra for compounds **1**–**3** (PDF)

Accession Codes

CCDC 2226694 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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